Identification of Overlapping HLA Class I–restricted Cytotoxic T Cell Epitopes in a Conserved Region of the Human Immunodeficiency Virus Type 1 Envelope Glycoprotein: Definition of Minimum Epitopes and Analysis of the Effects of Sequence Variation

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Summary

Although the immunologic basis of protective immunity in human immunodeficiency virus type 1 (HIV-1) infection has not yet been defined, virus-specific cytotoxic T lymphocytes (CTL) are likely to be an important host defense and may be a critical feature of an effective vaccine. These observations, along with the inclusion of the HIV-1 envelope in the majority of vaccine candidates presently in clinical trials, underscore the importance of the precise characterization of the cellular immune responses to this protein. Although humoral immune responses to the envelope protein have been extensively characterized, relatively little information is available regarding the envelope epitopes recognized by virus-specific CTL and the effects of sequence variation within these epitopes. Here we report the identification of two overlapping CTL epitopes in a highly conserved region of the HIV-1 transmembrane envelope protein, gp41, using CTL clones derived from two seropositive subjects. An eight–amino acid peptide was defined as the minimum epitope recognized by HLA-B8–restricted CTL derived from one subject, and in a second subject, an overlapping nine–amino acid peptide was identified as the minimal epitope for HLA-B14–restricted CTL clones. Selected single amino acid substitutions representing those found in naturally occurring HIV-1 isolates resulted in partial to complete loss of recognition of these epitopes. These data indicate the presence of a highly conserved region in the HIV-1 envelope glycoprotein that is immunogenic for CTL responses. In addition, they suggest that natural sequence variation may lead to escape from immune detection by HIV-1-specific CTL. Since the region containing these epitopes has been previously shown to contain an immunodominant B cell epitope and also overlaps with a major histocompatibility complex class II T cell epitope recognized by CD4⁺ CTL from HIV-1 rgp160 vaccine recipients, it may be particularly important for HIV-1 vaccine development. Finally, the identification of minimal CTL epitopes presented by class I HLA molecules should facilitate the definition of allele-specific motifs.

The extensive sequence diversity exhibited by HIV-1, especially in the envelope glycoprotein, represents a considerable challenge to the development of a vaccine against this retrovirus and may contribute to the ultimate failure of the immune system in HIV-1 seropositive subjects to contain viral replication. Detailed study of the recognition of HIV-1 epitopes by the human immune system may therefore facilitate the development of an HIV-1 vaccine and advance our understanding of the pathogenesis of AIDS. Although a considerable amount is known about humoral immune responses

to the HIV-1 envelope, our knowledge of specific epitopes recognized by cellular immune responses, and in particular epitopes recognized by CTL, remains incomplete. CTL are an important component of the host immune response against many viral infections, and the observations that CTL inhibit HIV-1 replication in vitro (1) and a decline in HIV-1-specific CTL activity may be associated with clinical progression to AIDS (2) suggest that HIV-1-specific CTL may play an important role in the inhibition of viral replication in vivo. These considerations have prompted us to perform a detailed examination of the CTL response to the HIV-1 envelope in infected persons.

Identification of CTL epitopes in the HIV-1 envelope gly-

coprotein has been complicated by the presence of both HLA-restricted and non–HLA-restricted envelope-specific cytolytic cells in PBMC (3–7). The evaluation of envelope-specific CTL responses using CTL clones should circumvent this problem and allow precise characterization of epitopes recognized by HLA-restricted CTL (8, 9). In the present study, we have identified CTL epitopes in the HIV-1 envelope glycoprotein using CD8⁺ CTL clones derived from two HIV-1 seropositive subjects. CTL epitopes restricted by two different HLA class I alleles were mapped to a 10–amino acid (aa)[1] conserved region of gp41. Further fine mapping demonstrated that this area contained an eight-aa HLA-B8-restricted CTL epitope and an overlapping nine-aa HLA-B14-restricted CTL epitope. Analysis of the effects of aa substitutions corresponding to natural sequence variation was performed for both CTL epitopes and demonstrated that a single aa change could lead to a loss of CTL recognition. These results support the hypothesis that sequence variation in HIV-1 may contribute to the evolution of virus variants that are no longer recognized by the host immune response.

Materials and Methods

Subjects. Two HIV-1 seropositive subjects who were known to have significant envelope-specific CTL activity were selected for study (8, 9). Both subjects gave written informed consent and the study was approved by the Massachusetts General Hospital Human Studies Committee. During this study, both subjects were asymptomatic; CD4⁺ lymphocytes were 145/mm³ in subject 010-035i and 900/mm³ in subject 010-115i. Subject 010-035i was referred to as subject 63 in a previous report from this laboratory (8).

Cell Lines. EBV-transformed B lymphoblastoid cell lines (B-LCL) were established and maintained as described previously (4) in RPMI 1640 (Sigma Chemical Co., St. Louis, MO) containing 20% (vol/vol) heat-inactivated FCS (Sigma Chemical Co.). RPMI 1640 used for all cell lines was supplemented with L-glutamine (2 mM), penicillin (50 U/ml), streptomycin (50 µg/ml), and Hepes (10 mM). Allogeneic B-LCL were also obtained from the American Society for Histocompatibility and Immunogenetics B cell line repository. All B-LCL were free of mycoplasma infection by standard culture techniques.

HLA Typing. HLA typing was performed by the Massachusetts General Hospital Tissue Typing Laboratory using standard serological techniques.

Recombinant Vaccinia Viruses. Preparation of recombinant vaccinia viruses expressing serial truncations of the HIV-1 envelope glycoprotein has been previously described (10). In brief, a series of truncated envelope genes were prepared from the BH8 isolate of HIV-1 (11) by digestion at unique restriction enzyme sites, and recombinant vaccinia viruses generated using homologous recombination. The recombinant vaccinia virus vAbt141 expressing the full-length p55 gag protein (12) was kindly provided by Drs. Gail Mazzara and Dennis Panicali (Therion Biologics Inc., Cambridge, MA). Recombinant vaccinia viruses expressing the HIV-1 reverse transcriptase (RT) (VCF21) (13) and the control *lacZ* (VSC8) genes were also used in these studies.

Synthetic HIV-1 Peptides. Synthetic peptides corresponding to the HIV-1 envelope PV22 sequence (14) were synthesized by Multiple Peptide Systems (San Diego, CA) using *t*-butoxycarbonyl NH₂-protected aa and 4-methylbenzhydramine resin (15). Peptides were cleaved from the resin with anhydrous hydrogen fluoride, washed with ether, extracted with 10% acetic acid, and evaluated for purity by reverse-phase analytical HPLC. Peptides consisted of a series of peptides 25 aa in length and overlapping by eight aa, and were synthesized as COOH-terminal amides, unless otherwise noted. All aa are numbered as indicated for the HXB2R sequence (16) and references to other peptides and epitopes have been modified accordingly. Peptide gp41/570 (numbering of NH₂-terminal aa changed to be consistent with Myers et al. [16]) was obtained from American BioTechnologies (Cambridge, MA). Peptide CP3 was provided by Repligen Corp. (Cambridge, MA), and peptide DP31 was donated by Drs. Kent Weinhold and Stefano Butto (Duke University, Durham, NC). Additional smaller peptides (8–15 aa) were synthesized for fine mapping using a technique similar to that described above (17). Lyophilized peptides were reconstituted at 2 mg/ml in sterile distilled water with 10% DMSO (Sigma Chemical Co.) with or without 1 mM dithiothreitol (Sigma Chemical Co.).

Isolation of HIV-1 Envelope-specific CTL Clones. CTL clones were isolated and maintained as described previously (8, 9). Briefly, PBMC obtained from seropositive subjects by separation on Ficoll-Hypaque (Sigma Chemical Co.) were incubated at 50 or 25 cells per well in 96-well plates with 200 µl of feeder cell solution containing 10⁶/ml irradiated allogeneic PBMC from HIV-1 seronegative subjects in RPMI 1640 with 10% heat-inactivated FCS (R10) supplemented with 100 U/ml of human rIL-2 (Hoffman-La Roche, Nutley, NJ; DuPont Co., Wilmington, DE; or Boehringer Mannheim Biochemicals, Indianapolis, IN). The CD3-specific mAb 12F6 (18) was added at 0.1 µg/ml as a stimulus to T cell proliferation. After 2–3 wk, cells from wells demonstrating growth were then transferred to 24-well plates and restimulated by adding 1 ml of rIL-2-containing medium with irradiated allogeneic PBMC (10⁶/ml) and anti-CD3 mAb (0.1 µg/ml). Approximately 2 wk later, clones were screened for CTL activity against autologous targets expressing the HIV-1 envelope glycoprotein or *lacZ* control. Clones exhibiting envelope-specific CTL activity were then restimulated every 10–14 d with anti-CD3 mAb and irradiated allogeneic PBMC.

Production of an Envelope-specific CTL Line. An envelope peptide-specific CTL line was derived using in vitro stimulation of PBMC obtained from subject 010-035i with an autologous, peptide-sensitized CD4⁺ T lymphocyte clone. CD4⁺ T lymphocyte clones were prepared by plating PBMC from subject 010-035i at limiting dilution in the presence of the bispecific mAb CD3,8 (18), irradiated allogeneic PBMC from HIV-1 seronegative donors, and R10 with rIL-2 (100 U/ml). The bispecific mAb CD3,8 leads to the selective expansion of CD4⁺ T lymphocytes by the lysis of CD8⁺ lymphocytes via redirected cytotoxicity and the concurrent activation of CD3⁺CD4⁺ lymphocytes (18). Wells exhibiting growth after 2 wk were restimulated using irradiated PBMC from seronegative donors and the CD3-specific mAb 12F6 as described below. Cell lines obtained using this technique were screened for CD4⁺ expression by flow cytometry, and cell lines with >98% CD4 expression were maintained with restimulation as described for CTL clones every 2–3 wk. These cells were then sensitized with a synthetic peptide (env/128) containing a CTL epitope by incubating them with 100 µg/ml of peptide for 2 h, washing twice with RPMI 1640, and then irradiating with 3,000 rad. Primary stimulation was carried out with peptide-sensitized CD4⁺ lymphocytes mixed

at a 1:1 ratio with fresh PBMC from subject 010-035i at a final concentration of PBMC in R10 of 4–6 × 10⁶/ml. After 4–7 d, rIL-2 was added to produce a final concentration of 10 U/ml. The peptide-specific CTL line was maintained by restimulation every 7–14 d with irradiated HIV-1 seronegative allogeneic PBMC and autologous peptide-sensitized, irradiated CD4⁺ lymphocytes in R10 supplemented with 10 U/ml rIL-2.

Flow Cytometry Analysis. Cells to be analyzed were incubated with a fluorescent conjugated anti-CD4 mAb, anti-CD8 mAb, or a similarly labeled control mAb (Coulter Electronics, Hialeah, FL) and analyzed with a FACScan® flow cytometer (Becton Dickinson & Co., Mountain View, CA) as previously described (9).

Cytotoxicity Assay. Target cells consisted of a B-LCL infected with recombinant vaccinia or preincubated with synthetic HIV-1 peptides. Vaccinia-infected targets were prepared by incubating 2.5–10 × 10⁶ B-LCL in log-phase growth with recombinant vaccinia at 1–2 PFU/cell for 16 h at 37°C. Cells were then labeled with 100–150 μCi of Na₂(⁵¹CrO₄) (New England Nuclear, Boston, MA) for 45–60 min and washed three times with R10. Peptide-coated targets were obtained by incubating 2–3 × 10⁶ B-LCL with peptide for 60 min during ⁵¹Cr labeling. Cytolytic activity was determined in a standard ⁵¹Cr-release assay (4) using 10⁴ targets per well. Plates were incubated in a humidified incubator at 37°C for 4 h (CTL clones) or 5 h (peptide-specific CTL line). All assays were performed in duplicate. Supernatants were then harvested and counted on a Cobra Gamma Counter (Packard Instrument Co., Inc., Downers Grove, IL), and percent lysis was determined from the formula: 100× [(experimental release − spontaneous release)/(maximum release − spontaneous release)]. Maximum release was determined by lysis of targets in water or detergent (1% Triton X-100; Sigma Chemical Co.). Spontaneous release was <30% of maximal release for all reported assays.

Results

Isolation of Envelope-specific CTL Clones. PBMC from two HIV-1 seropositive subjects were plated at limiting dilution in the presence of a CD3-specific mAb, irradiated PBMC from seronegative subjects, and rIL-2. Envelope-specific CTL were

Table 1. *Cytolytic Activity of HIV-1 Envelope-specific CTL Clones*

Subject	Clone	Percent specific lysis*			
		lac	gag	RT	Env
010-035i	35D45	2	2	0	72
010-115i	115G11	0	2	0	58
	115H10	1	0	0	73
	115J7	0	3	0	71
	115N10	0	0	0	72

* Target cells were prepared from autologous EBV-transformed B lymphoblastoid cell lines infected with recombinant vaccinia virus expressing the control *lacZ* gene (lac), HIV-1 p55 *gag* (gag), reverse transcriptase (RT), or envelope (Env) proteins. The E/T ratio was 10:1 for all clones.

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typically identified in 0.25–0.75% of all wells and comprised ~30–50% of all HIV-1-specific CTL. Five envelope-specific CTL clones, which were stable in long-term culture and could be fully characterized, are described in this paper. All were highly specific for the HIV-1 envelope (Table 1) and were found to be CD4⁻CD8⁺ by flow cytometry (data not shown).

Envelope-specific CTL Clones Are HLA Class I Restricted. The HLA restriction of envelope-specific lysis was defined for each CTL clone using a panel of allogeneic target cells matched at one or more class I alleles. Consistent with their CD8⁺ phenotype, all CTL clones were found to be HLA class I restricted. Clone 35D45 from subject 010-035i was restricted by the HLA-B8 molecule (Fig. 1). Testing of all four clones from subject 010-115i against allogeneic targets infected with recombinant vaccinia virus demonstrated lysis of targets matched at HLA-B14, either in the presence or absence of the Cw8 allele (Table 2). To further exclude restriction by the Cw8 allele, which is in dysequilibrium linkage with B14 (19), clone 115H10 was also tested against other allogeneic B-LCL and found to lyse additional target cells that express B14 but not Cw8 (data not shown).

Mapping of Epitope Specificity Using Recombinant Vaccinia Expressing Truncated HIV-1 Envelope Proteins. Initial mapping of the epitope specificity of envelope-specific CTL clones was performed using a panel of autologous target cells infected with recombinant vaccinia vectors expressing serial truncations of the HIV-1 envelope. Clone 35D45 and clones 115H10, 115J7, and 115N10 lysed target cells expressing envelope constructs containing the NH₂-terminal 635 aa of the HIV-1 envelope (vPE16, vPE17, and vPE18), but did not recognize targets expressing the 393 aa or shorter truncations (vPE20, vPE21, vPE22) (Fig. 2), identifying the region of aa 393–635 as containing the epitopes recognized by these clones. Clone 115G11 was not tested against targets expressing truncated
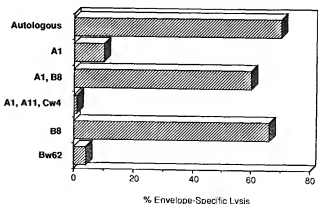

Figure 1. The CTL clone 35D45 is restricted by the HLA-B8 molecule. Target cells consisted of autologous or allogeneic B-LCL matched at the indicated HLA alleles, which were infected with recombinant vaccinia virus expressing either the HIV-1 envelope glycoprotein or the *lacZ* control. Targets were incubated with effector cells at an E/T ratio of 2.5:1. Envelope-specific lysis was calculated for each target tested by subtracting the specific lysis of targets infected with the control vaccinia virus from the specific lysis of envelope-expressing targets. Lysis of control targets was <12% for all assays.

Table 2. *Envelope-specific CTL Clones from Subject 010-115i Are HLA Class I Restricted*

Shared HLA alleles of target cell[‡]	Envelope-specific lysis[*]			
	115G11	115H10	115J7	115N10
Autologous	28	53	50	53
A2	2	1	0	0
A28	0	0	0	0
B14, Cw8	13	16	18	17
B14	21	53	51	ND
Bw52	0	0	0	0

[*] All CTL clones were incubated with target cells at an E/T ratio of 10:1. Envelope-specific lysis was calculated for each target tested by subtracting the specific lysis of lacZ-expressing targets from the specific lysis of envelope-expressing targets. Lysis of lacZ-expressing target cells was <2% for all targets.

[‡] Targets consisted of autologous or allogeneic B-LCL matched at indicated HLA antigens infected with recombinant vaccinia virus expressing either the HIV-1 envelope or *lacZ* control.

envelope proteins, but the specificity of this clone was subsequently defined using peptide-sensitized target cells (see below).

Identification of Envelope Peptides Recognized by Envelope-specific CTL Clones. Epitope specificity was then defined for each CTL clone using autologous target cells incubated with a series of overlapping peptides, 25 aa in length with an eight aa overlap, which spanned aa 393–635. Remarkably, the HLA-B8-restricted CTL clone 35D45 from subject 010-035i and all of the HLA-B14-restricted CTL clones from subject 010-115i recognized the same envelope peptide env/128, aa 575–599, sequence QLQARILAVERYLKDQQLLGIWGCS (single letter code) with no recognition of any other synthetic peptide spanning this sequence (Table 3).

Generation of an Envelope-specific CTL Line from Subject 010-035i. Although preliminary fine mapping of the epitope specificity of CTL clone 35D45 was performed (see below), the limited ability to propagate clone 35D45 precluded definitive fine mapping of the epitope specificity of this clone. Attempts to produce a CTL line specific for peptide env/128 from subject 010-035i by in vitro stimulation of PBMC with

synthetic peptide as previously described for influenza (20) were unsuccessful (data not shown). To fine map the HLA-B8-restricted CTL epitope, an envelope-specific CTL line was therefore derived from subject 010-035i using an autologous, envelope peptide–sensitized CD4^{+} T lymphocyte clone. After primary and secondary stimulations of PBMC with an irradiated CD4^{+} T lymphocyte clone sensitized with peptide env/128, a CTL line was derived that was highly specific for the sensitizing peptide (Fig. 3). This CTL line also recognized autologous target cells infected with recombinant vaccinia virus expressing the HIV-1 envelope (data not shown). CTL lines generated using this technique maintained highly specific cytolytic activity for peptide env/128 for 8–12 wk when restimulated every 7–10 d with peptide-sensitized autologous CD4^{+} lymphocytes and irradiated allogeneic PBMC. Experiments using peptide-sensitized allogeneic target cells confirmed that lysis by the 35/env/128 CTL line was restricted by the HLA-B8 allele, as was observed for clone 35D45 (data not shown).

HLA-B8- and B14-restricted CTL Recognize Overlapping Epitopes in gp41. Fine mapping of the specific epitopes recognized by gp41-specific CTL was performed by analyzing the lysis of target cells sensitized with peptides containing sequences within peptide env/128 (aa 575–599). Initial epitope mapping revealed that both HLA-B8-restricted CTL (clone 35D45 and line 35/env128) and HLA-B14-restricted CTL (clones 115H10 and 115J7) recognized peptides that contained the common sequence ERYLKDQQLLG (aa 584–594) (Table 4). However, peptides gp41/576 and GGO6-15, which contained only the NH₂-terminal nine aa (ERYLKDQQL) of this shared sequence, were recognized by the HLA-B14-restricted CTL but not by the HLA-B8-restricted CTL, suggesting that two different overlapping CTL epitopes existed within this region.

To define precisely both the HLA-B8- and B14-restricted gp41 CTL epitopes, peptides with sequential single aa deletions were used to sensitize autologous target cells for lysis by CTL derived from both subjects. Deletions of a single aa at either end of the nine-aa sequence ERYLKDQQL (aa 584–592) resulted in loss of recognition by the HLA-B14-restricted clones 115H10 and 115J7 (Table 4), identifying this sequence as the minimum epitope required for recognition by these clones. Testing of the remaining gp41-specific clones obtained from subjects 010-115i, 115G11, and 115N10 against the same panel of peptides produced an identical pattern of epitope specificity
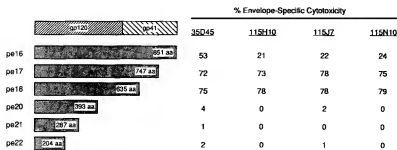
Figure 2. Epitope mapping of envelope-specific CTL clones using recombinant vaccinia viruses expressing serial truncations of the HIV-1 envelope. Autologous B-LCL were infected with recombinant vaccinia expressing serial truncations of the HIV-1 envelope or the *lacZ* control. The length of HIV-1 envelope expressed by each construct is indicated. Clones were incubated with target cells at several E/T ratios, and representative data are shown for clone 35D45 at an E/T ratio of 5:1 and clones 115H10, 115J7, and 115N10 at an E/T ratio of 10:1. Envelope-specific lysis was calculated for each target by subtracting the specific lysis of targets infected with the control vaccinia virus from the specific lysis of envelope-expressing targets.

Table 3. *Epitope Mapping of Envelope-specific CTL Clones*

Peptide	Amino acids	Percent specific lysis[*]			
		35D45	115H10	115J7	115N10
env/117	388–412	2	0	0	1
env/118	405–429	2	1	1	0
env/119	422–446	0	0	1	1
env/120	439–463	1	0	1	0
env/121	456–480	2	0	1	1
env/122	473–497	1	1	0	1
env/123	490–514	2	0	1	0
env/124	507–531	2	0	0	0
env/125	524–548	1	0	1	1
env/126	541–565	1	0	0	0
env/127	558–582	1	0	0	0
env/128	575–599	66	38	22	39
env/129	592–616	0	0	0	1
env/130	609–633	0	0	0	0

[*] CTL clones were incubated with autologous target cells at an E/T ratio of 4:1 (35D45) or 10:1 (115H10, 115J7, 115N10). Peptide-sensitized target cells were prepared by incubating autologous B-LCL with 100 μg/ml of the indicated peptide for 60 min during ^{51}Cr labeling. The sequence of peptide env/128 is QLQARILAVERYLKDQQLLGIWGCS.
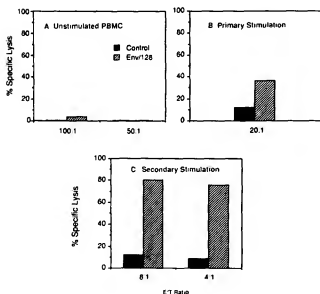
Figure 3. Generation of an HIV-1 envelope peptide–specific CTL line. PBMC from an HIV-1 seropositive subject (subject 010-035i) were used as effector cells either fresh (*A*) or after a single (*B*) or second (*C*) stimulation with a peptide env/128–sensitized irradiated autologous CD4⁺ lymphocyte clone and irradiated allogeneic PBMC. Targets consisted of autologous B-LCL incubated either with the peptide env/128 or culture medium alone (control).

(data not shown). In a similar fashion, deletions of single aa identified the eight-aa sequence YLKDQQLL (aa 586–593) as the putative minimum epitope for the HLA-B8–restricted CTL line 35/env/128 (Table 4). The octamer corresponding to the HLA-B8–restricted gp41 minimum epitope, synthesized as a COOH-terminal acid, was able to sensitize target cells for lysis by the 35/env/128 CTL line at concentrations as low as 100 pmol (data not shown).

Effects of Sequence Variation on Recognition by HLA-B8- and B14-restricted CTL. Given the relatively high degree of variability in the HIV-1 envelope glycoprotein, we wished to assess the effects of sequence variation exhibited by diverse HIV-1 isolates on recognition by envelope-specific CTL. Peptides were synthesized that contained both the HLA-B8- and B14-restricted gp41 CTL epitopes and exhibited aa substitutions corresponding to sequenced HIV-1 isolates and the HIV-2 ROD isolate. The introduction of a conservative substitution (Arg for Lys) at position 588 had no significant effect on recognition of the variant peptide by B14-restricted CTL, but resulted in a partial loss of lysis by B8-restricted CTL (Fig. 4). However, the nonconservative substitution of Gln for Lys at this same position abolished recognition by both HLA B8 and B14-restricted CTL. Interestingly, the introduction of a second aa substitution, Arg for Gln substitution at position 591, resulted in recognition by both HLA-B8-

Table 4. *Fine Mapping of CTL Epitopes in gp41 Recognized by HLA-B8- and B14-restricted CTL*

Peptide	Sequence	Percent specific lysis[*]			
		35D45	35/env128	115H10	115J7
env/128	QLQARILAVERYLKDQQLLGIWGCS	74	37	38	31
gp41/576	LQARILAVERYLKDQQL	7	5	77	64
GGO6-15	LQARILAVERYLKDQQL (GC)	10	10	62	67
GGO6-16	AVERYLKDQQLLGIWGC	90	40	44	50
CP3	ILAVERYLKDQQLLG (C)	81	66	34	22
DP31	ERYLKDQQLLGIWGCSGKLICG	74	32	83	59
env/128A	AVERYLKDQQLLGIW	ND	34	48	43
env/128B	AVERYLKDQQLLGI	ND	43	46	46
env/128C	AVERYLKDQQLLG	ND	39	58	26
env/128D	AVERYLKDQQLL	ND	35	57	40
env/128E	AVERYLKDQQL	ND	6	71	77
env/128F	AVERYLKDQQ	ND	0	7	3
env/128H	AVERYLKD	ND	1	7	6
env/128I	VERYLKDQQLLGIW	ND	22	70	62
env/128J	ERYLKDQQLLGIW	ND	34	83	83
env/128K	RYLKDQQLLGIW	ND	35	2	7
env/128L	YLKDQQLLGIW	ND	39	0	4
env/128M	VERYLKDQQLL	ND	39	50	50
env/128N	VERYLKDQQL	ND	4	83	81
env/128O	ERYLKDQQL	ND	8	87	80
env/128P	VERYLKDQQ	ND	0	3	1

[*] Effector cells consisted of the HLA-B8-restricted CTL clone 35D45 and CTL line 35/env128 derived from subject 010-035i or the HLA-B14-restricted CTL clones 115H10 and 115J7 derived from subject 010-115i. Each effector cell was tested at multiple E/T ratios. Representative data are shown for the CTL clone 35D45 at an E/T of 2:1, the CTL line 35/env128 at an E/T ratio of 5:1, and for the CTL clones 115H10 and 115J7 at an E/T ratio of 3:1. Targets consisted of autologous B-LCL incubated with the indicated peptide for 60 min at a final concentration of 100 μg/ml during ⁵¹Cr labeling. Amino acids enclosed in parentheses indicate peptide amino acids that do not correspond to the HIV-1 sequence.

and B14-restricted CTL, although sensitization required higher concentrations than the corresponding HXB8 peptide. The observation that a second aa substitution can restore recognition of a variant peptide has also been made for a murine cytomegalovirus epitope (21). The peptide corresponding to the HIV-2 ROD sequence was not able to sensitize autologous target cells for lysis by CTL derived from either subject.

Discussion

These data identify a highly conserved region of the gp41 transmembrane protein as a target for class I–restricted CTL and define within this region two distinct, overlapping T cell epitopes. Based on their recognition of synthetic peptides corresponding to natural sequence variation, CTL specific for these epitopes are able to recognize >90% of sequenced North American isolates (16). Structural requirements for the conservation of this region of HIV-1 may be related to functions of the gp41 protein or selection at the level of nucleic acid, as the region encoding these CTL epitopes corresponds

to the end of the rev-responsive element (22, 23). These findings of a cell-mediated response directed at conserved regions of the HIV-1 envelope are consistent with those of Earl et al. (10), who isolated CD8⁺ CTL that recognized divergent HIV-1 strains, although specific epitopes were not identified, and with those of other investigators who have documented T helper cell responses against conserved regions of the HIV-1 envelope (24). Type-specific CTL directed at highly variable regions of the HIV-1 envelope glycoprotein have also been identified (25, 26, and R. P. Johnson, C. Jassoy, and B. D. Walker, unpublished data), and the relative contribution of group-specific and type-specific anti–envelope CTL remains to be determined.

CTL responses directed at this conserved region of gp41 are likely to exist in a significant percentage of HIV-1 seropositive subjects. The HLA-B8 and B14 molecules are expressed on approximately 16% and 7% of the North American Caucasian population and 6% and 8% of North American black population, respectively (19). HLA-B14-restricted CTL clones that recognize aa 584–592 have been obtained to date from

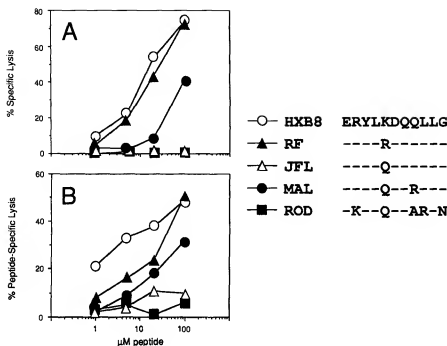|		
—○—	HXB8	ERYLKDQQLLG
—▲—	RF	----R------
—△—	JFL	----Q------
—●—	MAL	----Q--R---
—■—	ROD	-K--Q--AR-N

Figure 4. Effect of natural sequence variation on recognition of gp41-specific CTL. Autologous B-LCL were sensitized with the indicated concentration of peptide and incubated with either the B14-restricted CTL clone 115H10 (A) or the B8-restricted CTL line 35/env128 (B) at E/T ratios of 3:1 and 5:1, respectively. Peptide-specific lysis was calculated for the CTL line 35/env/128 by subtracting the lysis of unsensitized B-LCL from the lysis of peptide-sensitized targets, which ranged from 8% to 15%.

two other HIV-1 seropositive subjects, suggesting that recognition of this epitope is common in HLA-B14-positive subjects (R. P. Johnson, C. Jassoy, and B. D. Walker, unpublished data). In addition, a peptide including both HLA-B8-and B14-restricted gp41 epitopes corresponding to aa 580-600 of the HIV-1 envelope has been shown to bind to isolated HLA-A2 molecules in a solid phase assay, suggesting that the A2 allele may be able to present peptides from this region (27). Although the structural basis for overlapping T cell epitopes is not known, several examples have been reported recently, including MHC class I–restricted CTL epitopes reported in the HIV-1 nef (28, 29) and gag (9, 30) proteins, and MHC class II–restricted T cell epitopes in other systems (31–33).

It is important to note that our study differs from other recent reports on envelope-specific CTL (25, 34) in that CTL epitopes were identified in an inclusive fashion, rather than on the basis of screening against a subset of synthetic envelope peptides. In addition, effector cells used for the initial definition of the epitopes described here were not stimulated in vitro with viral antigen. Identification of T cell epitopes using synthetic peptides chosen on the basis of amphipathicity (25) or sequence conservation (34) may underestimate the complete repertoire of the anti-envelope CTL response. Moreover, characterization of CTL epitopes using effector cells produced by in vitro stimulation with irradiated HIV-1-infected lymphoblasts (34), a process that is able to induce CTL responses in HIV-1-seronegative subjects (2, 34, 35), raises the possibility that epitopes identified in this fashion may not accurately reflect epitopes recognized in vivo by HIV-1 seropositive subjects.

The region in gp41 containing the epitopes identified here appears to be important not only for HLA class I–restricted

cytotoxic responses, but also for a number of additional immune functions. The HLA-B8 (aa 586–593) and B14 (584–592)-restricted CTL epitopes partially overlap with an epitope (aa 579–590) recognized by CD4+ lymphocytes generated in seronegative subjects after immunization with a rgp160 vaccine (36). A peptide from gp41 containing the class I HLA-B8 and B14 CTL epitopes and the class II CD4 epitope has also been reported to induce proliferative responses in 24% of seropositive subjects (37). Association between class I and class II epitopes has also been noted for other HIV-1 epitopes (25). Interestingly, this region is also immediately adjacent to the immunodominant B cell epitope in gp41, aa 593–604 (38). However, this region of gp41 also includes a peptide that has been proposed to have potential immunosuppressive effects (39), and further studies are necessary to reconcile these observations.

An increasing body of evidence suggests that sequence variation within critical epitopes of HIV-1 may lead to virus variants that escape recognition by the host immune response (40, 41). HIV-1 genomes exhibit considerable sequence variation, even within relatively conserved proteins such as gag (42) and tat (43). However, only limited information exists regarding the impact of sequence variation on recognition by human HIV-1-specific CTL. Previous studies in a murine model have indicated that even a single aa change in the HIV-1 envelope may abrogate recognition by virus-specific CTL (44). For the two gp41 epitopes described in this report, a conservative aa substitution resulted in either no effect on recognition or a modest reduction of target lysis, while a single nonconservative substitution resulted in a complete loss of recognition. For other HIV-1 epitopes, conservative aa substitutions have also been observed to abolish lysis of target cells (36, R. P. Johnson and B. D. Walker, unpublished ob-

Table 5. *Comparison of Peptides Containing CTL Epitopes Presented by the HLA-B8 Molecules*

Antigen (aa coordinates)	Sequence*	Reference
HIV-1 gp41 (586–593)	Y L K D Q Q L L	This report
HIV-1 p17 (21–35)	L R P G G K K K Y K L K H I V	30
HIV-1 p17 (21–35)	L R P G G K K K Y K L K H I V	
HIV-1 p17 (21–35)	L R P G G K K K Y K L K H I V	
HIV-1 p24 (253–267)	N P P I P V G E I Y K R W I I	9, 53
HIV-1 p24 (253–267)	N P P I P V G E I Y K R W I I (L)	
HIV-1 p24 (323–337)	V Q N A N P D C K T I L K A L	30
HIV-1 p24 (323–337)	V Q N A N P D C K T I L K A L	
HIV-1 RT (160–184)	I E T V P V K L K P G M D G P K V K Q W P L T E E	8
HIV-1 RT (160–184)	I E T V P V K L K P G M D G P K V K Q W P L T E E	
EBV EBNA (334–353)	A W N A G F L R G R A Y G I D L L R T E	54
EBV EBNA (334–353)	A W N A G F L R G R A Y G I D L L R T E	

* Eight- or nine-aa regions aligned with the HLA-B8–restricted gp41 CTL epitope YLKDQQLL are shown in boldface and homologous aa are underlined. Alternative alignments are shown for each peptide. For one alignment of the p24 gag epitope 253–267, an additional aa corresponding to the natural sequence but not included in the sensitizing peptide is shown in parentheses. All HIV-1 peptides are numbered according to the HXB2R sequence (16).

servations). These results underscore the potential impact of sequence variation in HIV-1 to produce virus variants that can escape immune recognition.

In addition to allowing a precise assessment of the effects of sequence variation on recognition by CTL, the determination of minimum CTL epitopes may also facilitate the identification of allele-specific motifs. The minimum epitopes identified here of eight and nine aa for the HLA-B8- and B14-restricted gp41 epitopes are consistent with the recent characterization of naturally processed peptides of eight or nine aa in both mouse and humans (45–50) and the crystalline structure of the peptide binding pocket of the HLA-B27 molecule (51). The minimum epitopes defined in this study using serial truncations of synthetic peptides may well correspond to the naturally processed peptide, consistent with the ability of the octamer HLA-B8-restricted gp41 epitope to sensitize target cells at picomolar concentrations. Allele-specific motifs have been proposed for several mouse MHC types and the HLA-A2 and B27 alleles (47–49, 52). Comparison of the HLA-B8-restricted gp41 epitope 586–593 with peptides containing other HLA-B8-restricted CTL epitopes (8, 9, 30, 53, 54) reveals several potential alignments with shared characteristics at selected positions within an eight- to nine-aa region, including a hydrophobic residue (Ala, Leu, Iso, and Val) at position two, a positively charged aa at position 3, and aa with nonpolar or small polar side chains at the two COOH-terminal residues (Table 5). Definitive assignment of an HLA-B8-specific motif will require the determination of minimum effective epitopes for other HLA-B8-restricted epitopes and the use of competition experiments with variant peptides to define

aa that interact with either the TCR or HLA molecule (55–58). Additional HLA-B14-restricted CTL epitopes need to be identified in order to propose a motif for this allele. Precise definition of epitopes recognized by CTL clones may improve our ability to predict immunogenic sequences based on allele-specific motifs. The significance of such allele-specific motifs is further emphasized by the fact that an algorithm that was developed to identify T cell epitopes based on amphipathicity (59, 60) fails to identify epitopes described by other investigators (61) and those in this report.

Since the majority of HIV-1 vaccine candidates currently in clinical trials include the envelope protein as an immunogen (62, 63), further definition of the human CTL response to gp160 is likely to facilitate evaluation of immune responses in vaccine recipients. In the studies reported here, we have devised a technique whereby CTL can be expanded in vitro using stimulation with uninfected, irradiated, autologous CD4+ lymphocytes incubated with synthetic peptides as antigen-presenting cells. These data therefore indicate that CTL from HIV-1-infected persons can be expanded in vitro in an epitope-specific manner. This protocol has been successful in expanding CTL specific for other HIV-1 epitopes as well (data not shown), and may prove useful as a means of detecting low level or memory responses to HIV-1 proteins in HIV-1 vaccine recipients.

In summary, these studies provide evidence that the highly conserved gp41 transmembrane protein serves as an important target for class I-restricted CTL. The identification of two overlapping CTL epitopes in gp41, which are restricted by different HLA molecules, may be useful not only in subunit

vaccine design, but should also be helpful in defining CTL responses in HIV-1 vaccine recipients. The existence of CTL epitopes in the relatively conserved portions of the envelope glycoprotein suggests that vaccines capable of inducing CTL responses may be able to induce cross-reactive immunity. How-

ever, in light of the ability of single aa changes, even conservative substitutions, to abolish recognition by CTL, potential HIV-1 vaccines should be designed to induce cellular immune responses against multiple conserved regions of HIV-1.

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